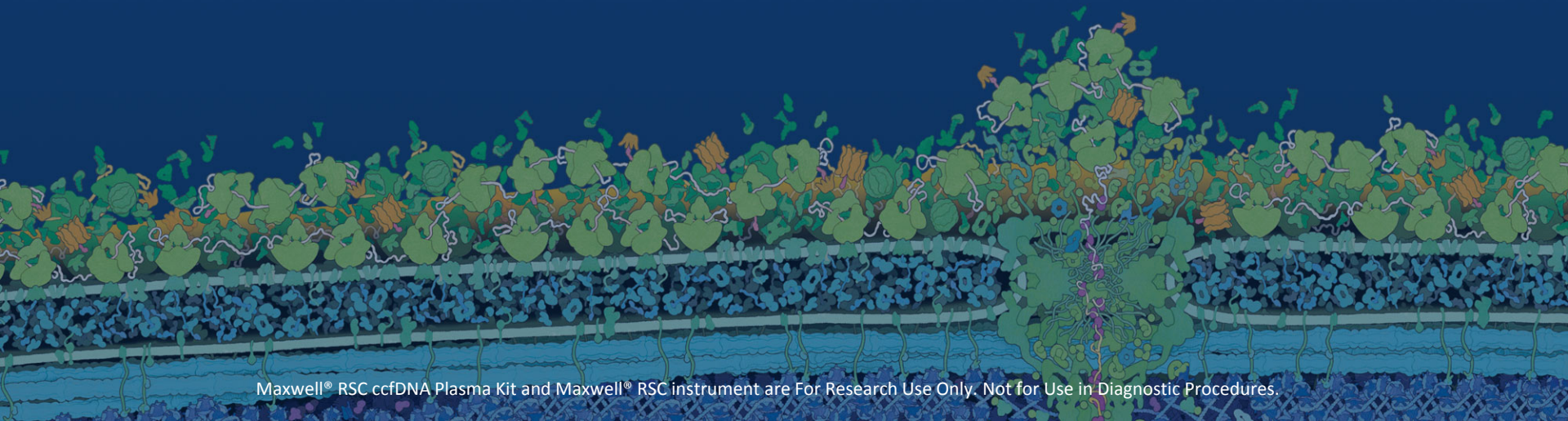
ccfDNA Webinar Series: The Basics and Beyond

Part 2

ccfDNA Workflows: Honing in on the Target

Douglas Horejsh



Disclaimer

All Promega products listed below and in this presentation are intended For Research Use Only and are Not for Use in Diagnostic Procedures.

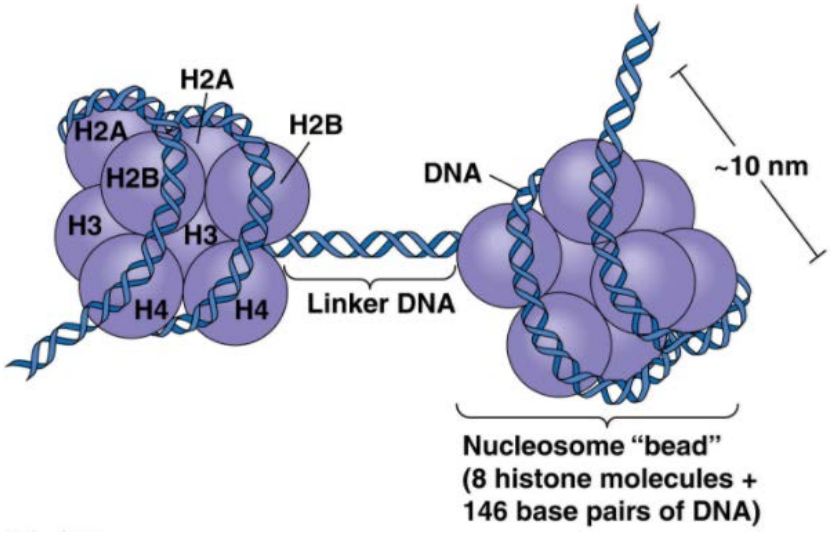
- Maxwell® RSC Instrument
- Maxwell® ccfDNA Kit
- Quantus™ Fluorometer
- Maxwell® LV Instrument
- Promega HSM 2.0
- Maxwell® HT System
- MaxPrep™ Liquid Handler
- Maxwell® HT Chemistries
- ProNex® Size-Selective Purification System

What will we discuss today?

- Challenges and solutions for ccfDNA purification
- Common methods used to quantify ccfDNA
- Evaluation of purification methods

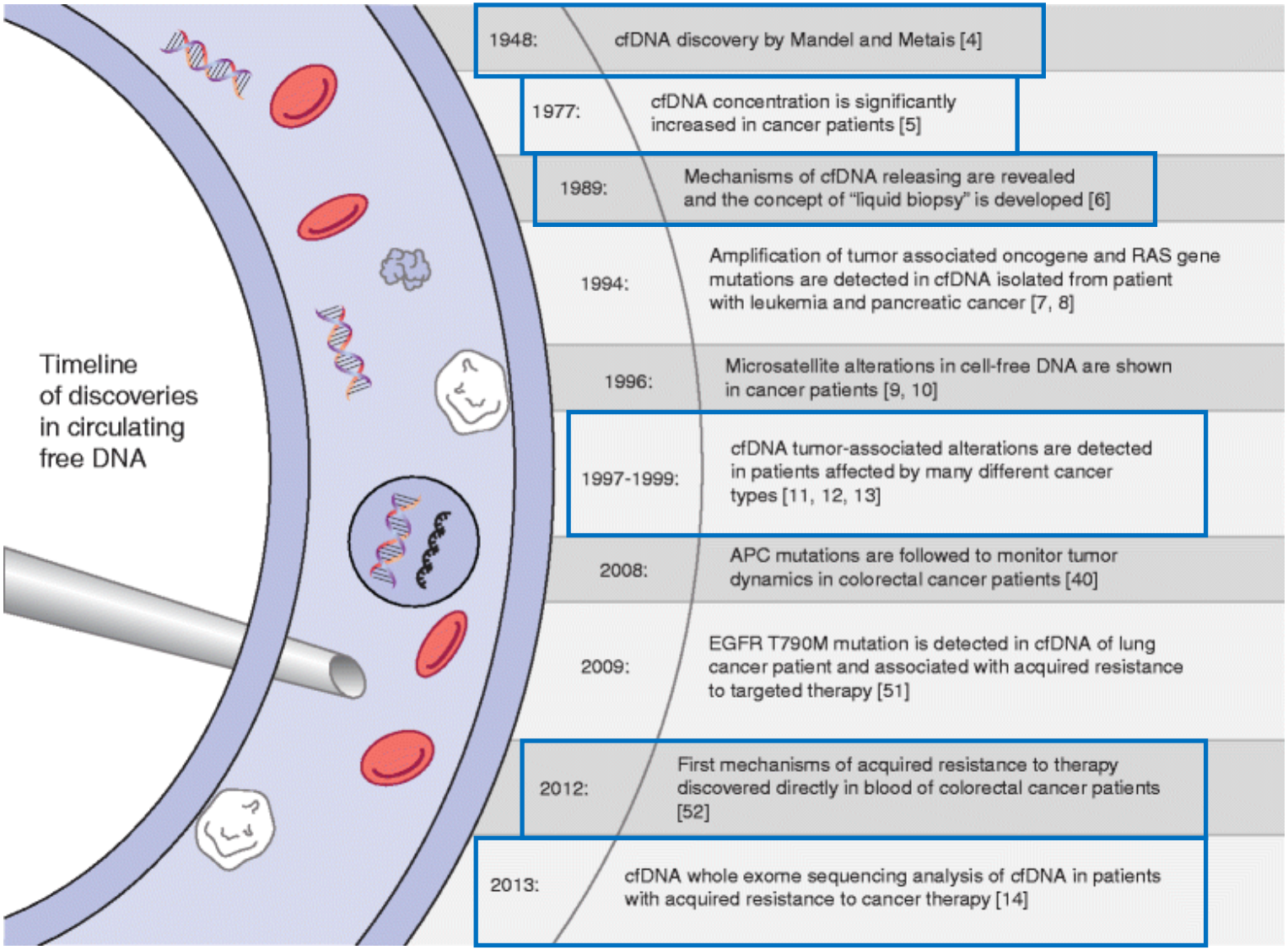
Circulating Cell-Free DNA (ccfDNA)

- **cfDNA, ctDNA, cfcDNA**
- **Nucleosomal in size: ~170bp**
  - Dimers and trimers can be seen
- **Dilute: 5-30ng per ml plasma**
  - Increases in cancer, trauma
  - Increased heart rate can increase levels
- **Rapidly turned over**
  - Half-life of 30 minutes
  - Allows “snapshot” of current genetic make up



Memorial University, Faculty of Science - Biology
http://www.mun.ca/biology/desmid/brian/BIOL2060/BIOL2060-18/18_21.jpg

ccfDNA Timeline



https://genomebiology.biomedcentral.com/articles/10.1186/s13059-014-0449-4

Challenges for Purifying ccfDNA

- Very dilute – low concentration
- Fragmented DNA
- Downstream assays are complex – need clean DNA
- Automated method to process preferred

Purification Systems for ccfDNA

- Phenol chloroform extraction

- Silica-based systems
  - Predominantly column-based chemistry
  - Protease K digestion step
  - May require carrier RNA

- Cellulose-based systems
  - No protease K step
  - No carrier RNA
  - Potential for automation

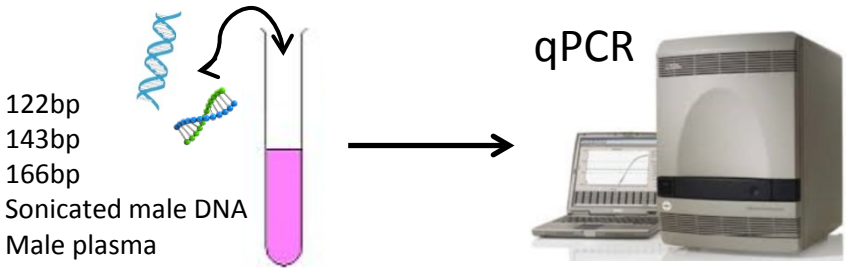
Evaluation of the Different Chemistries

Silica resin

- Higher yield
- Initial protease K step at 55ºC
- Pre-processing required
- Increased volumes

Cellulose resin

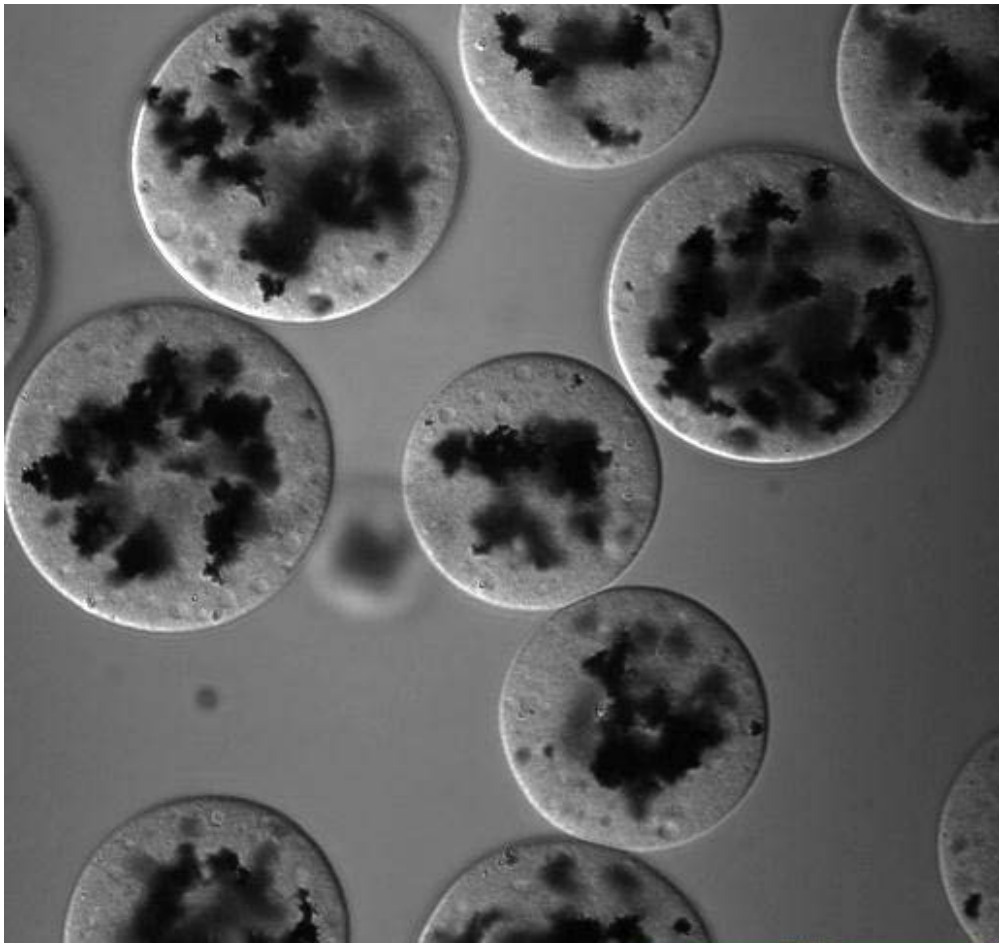
- No protease K step
- Could be completely automated



Silica Resin	Cellulose Resin
Industry standard	Proprietary
Proteinase K step	No protease step
Elevated temperatures	Room temperature
High maximum volume (4x)	Lower volume (2x)
Good Yield	Lower yield

Resins

- Novel resin
- Developed at Promega; internal capabilities
- Spherical particle with iron cores
- Less exposed iron



Automation method preferred

Reliable—Prefilled cartridges and preprogrammed methods means consistent purification

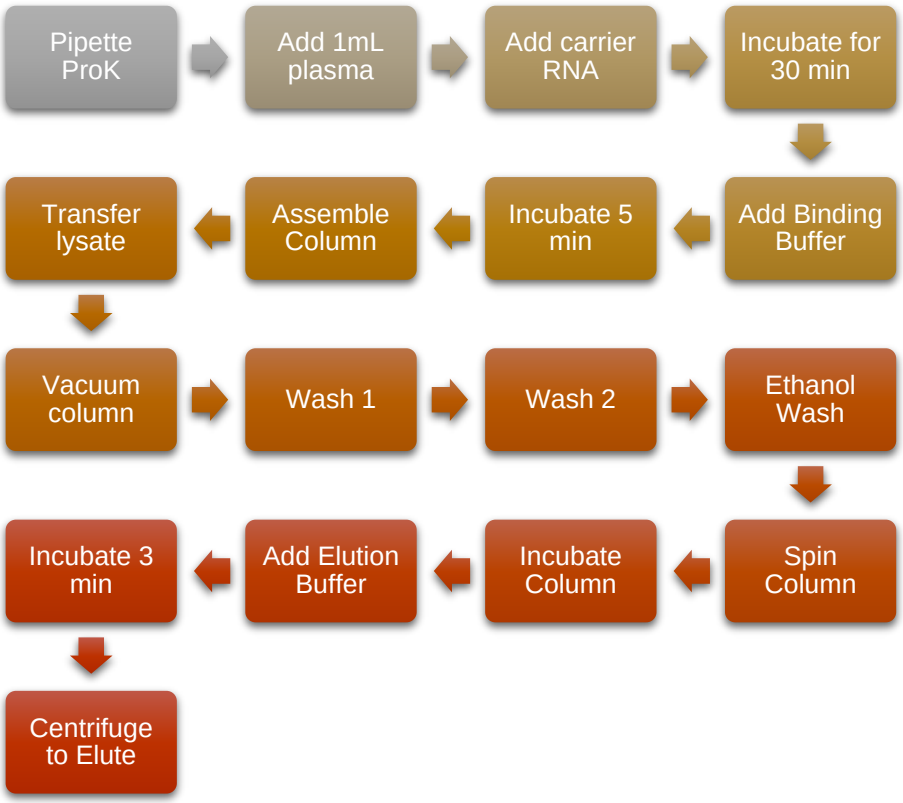
Fewer manual steps—Less hands-on time needed

Intuitive—Minimal training required. The instrument software guides users through a purification run

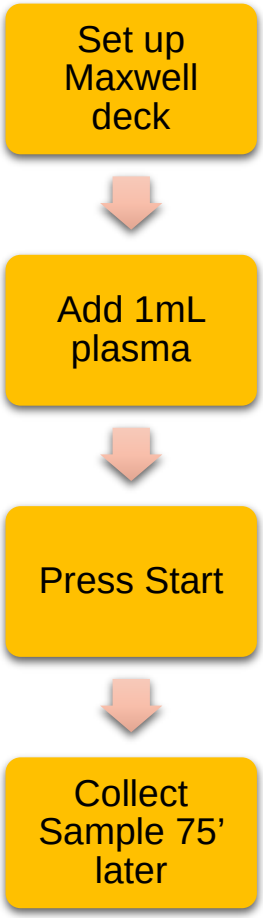
Efficient—Automated nucleic acid purification frees up your time, and the integrated Quantus™ Fluorometer streamlines the next quantitation step

Protocol for ccfDNA purification

Manual Silica Circulating Nucleic Acid



Maxwell® RSC ccfDNA Plasma Kit



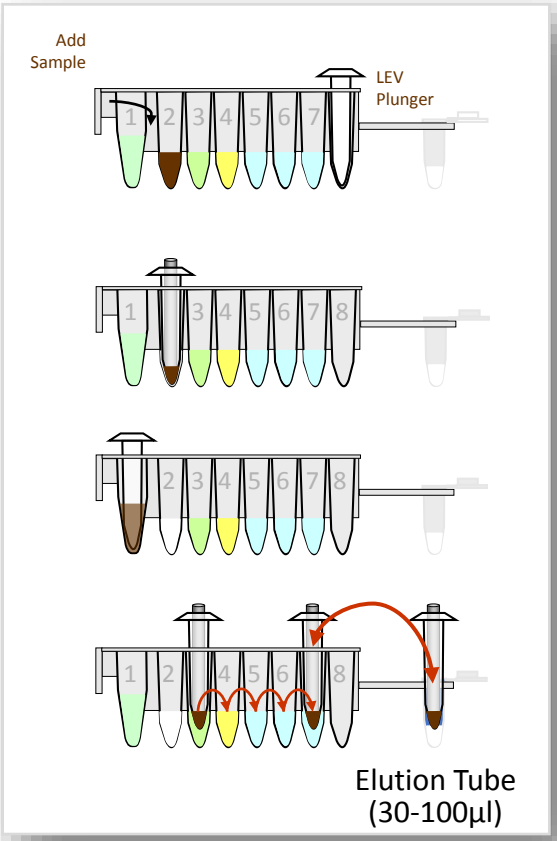
Maxwell® RSC Instrument
Easy-to-Use, Particle Mover Automation

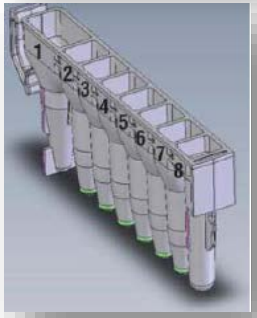
Mixing

Capture

Binding

Washing & Elution

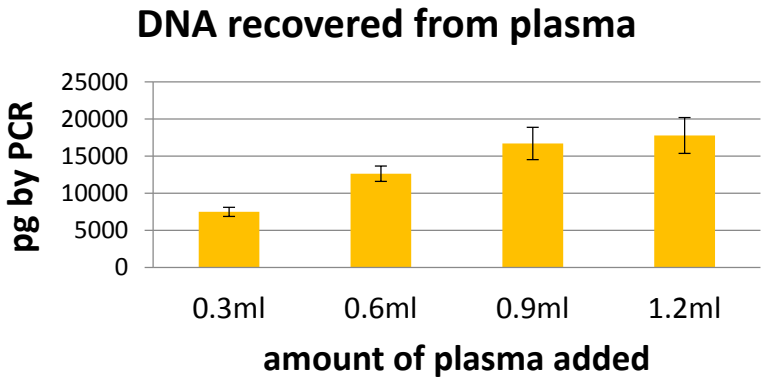


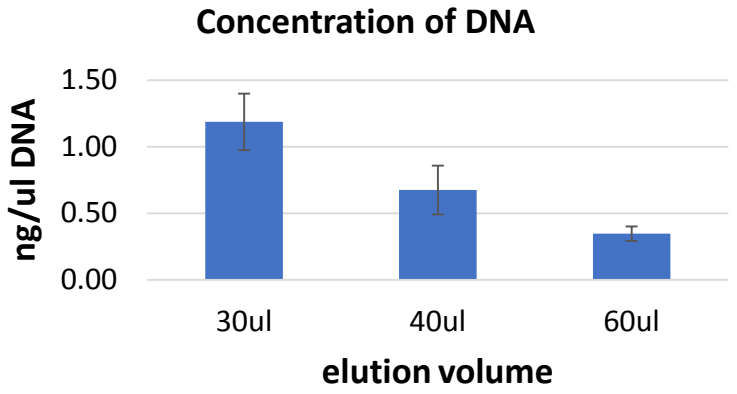


Parallel processing of 1-16 samples at a time

Maxwell® RSC ccfDNA Kit

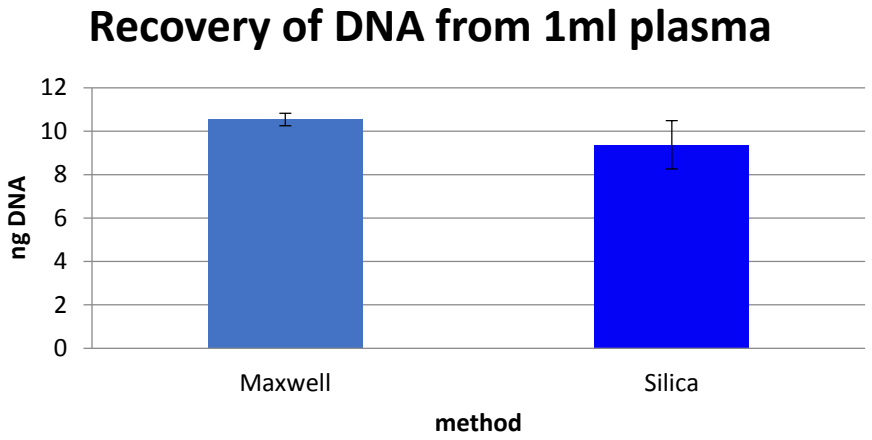
- RUO product for research labs
- Process up to 1ml plasma
- Completely automated
- 16 samples in 75 minutes
- Elute in 30 to 60ul buffer

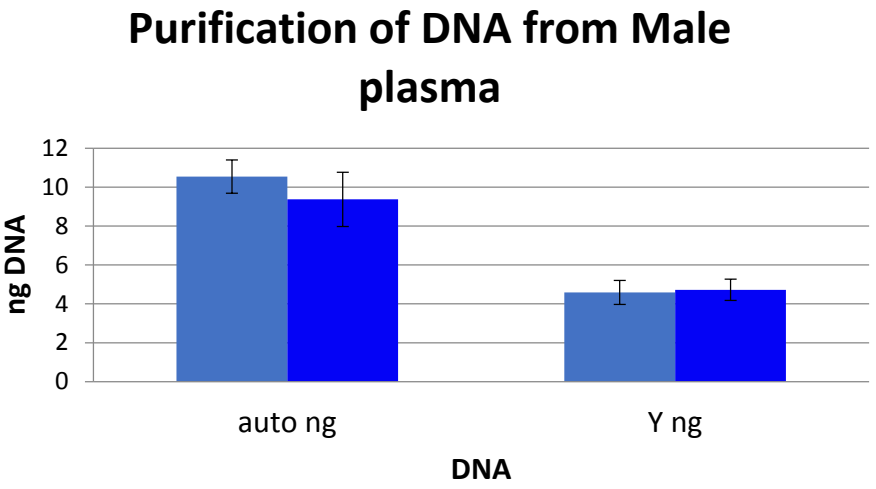




Comparison of Silica Method and Maxwell® Method

- Yields are equivalent when measured by qPCR
- Elution volumes are identical





Maxwell® LV

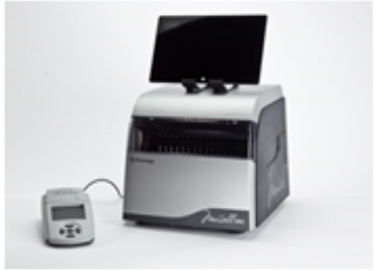
- Processes larger volumes of plasma
- Pre-processing: Binding of DNA to resin on HSM 2.0 or manual
- Sample is concentrated and transferred to Maxwell® Instrument

Binding/Concentration

Promega HSM 2.0

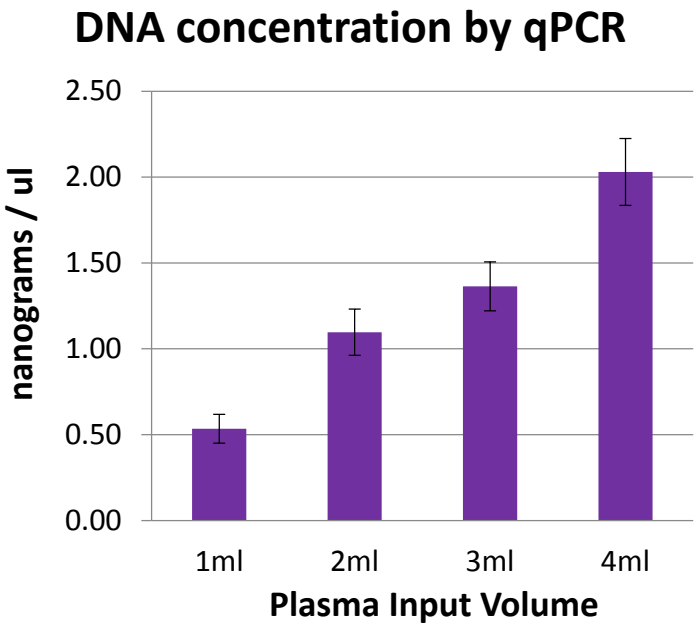
Transfer of particle-bound ccfDNA into Maxwell

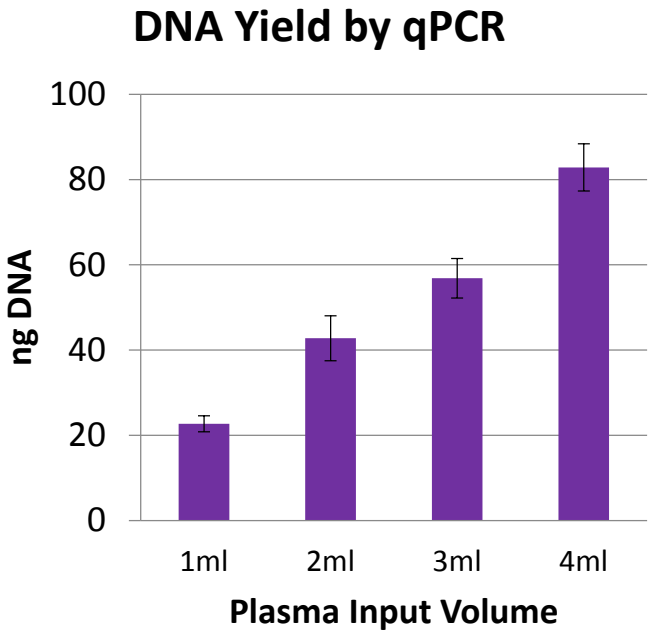
Wash/Elution



Maxwell® RSC

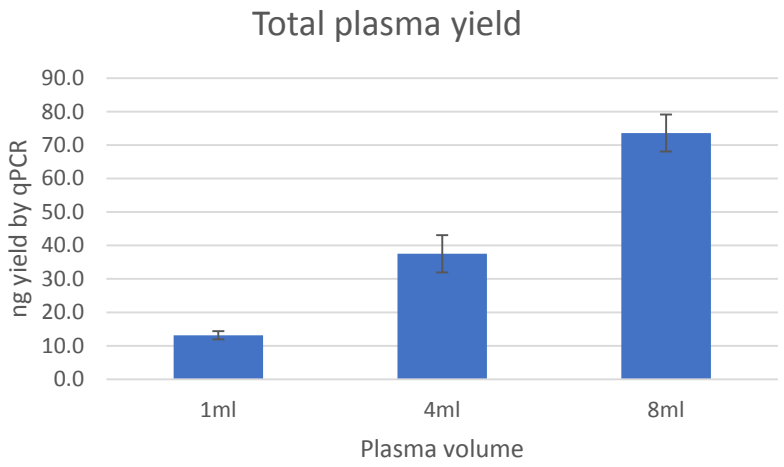
Maxwell® LV Results – Good Scalability

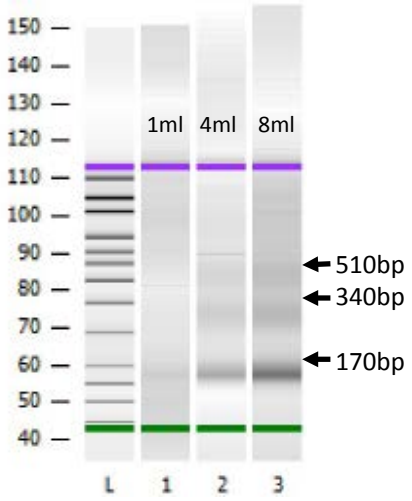




Results

- 8mL works in this system as well
- Tested up to 20mL of plasma input

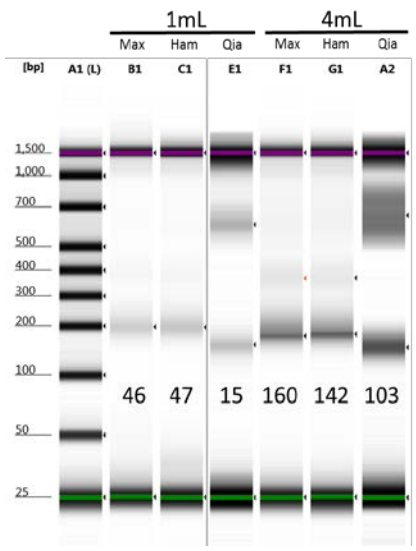




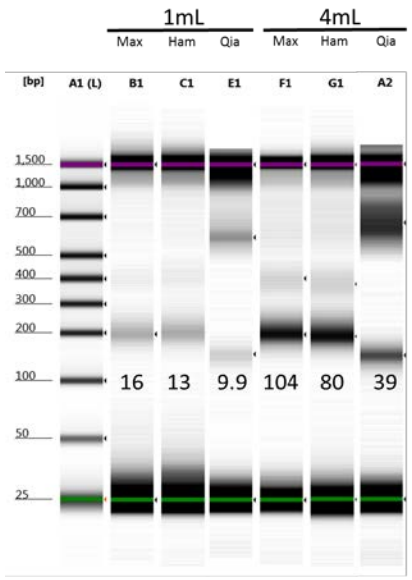
Comparison of 1mL and 4mL Runs of Maxwell® LV, Maxwell® HT and Qiagen

- Numbers below show estimated concentration in pg/uL for the 170bp band
- Qiagen numbers likely underestimated due to interference with lower and upper markers

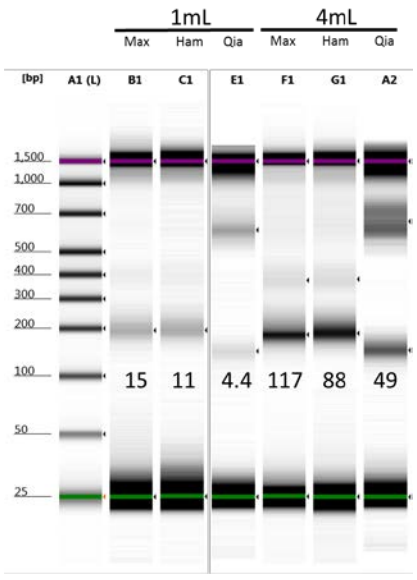
Donor C



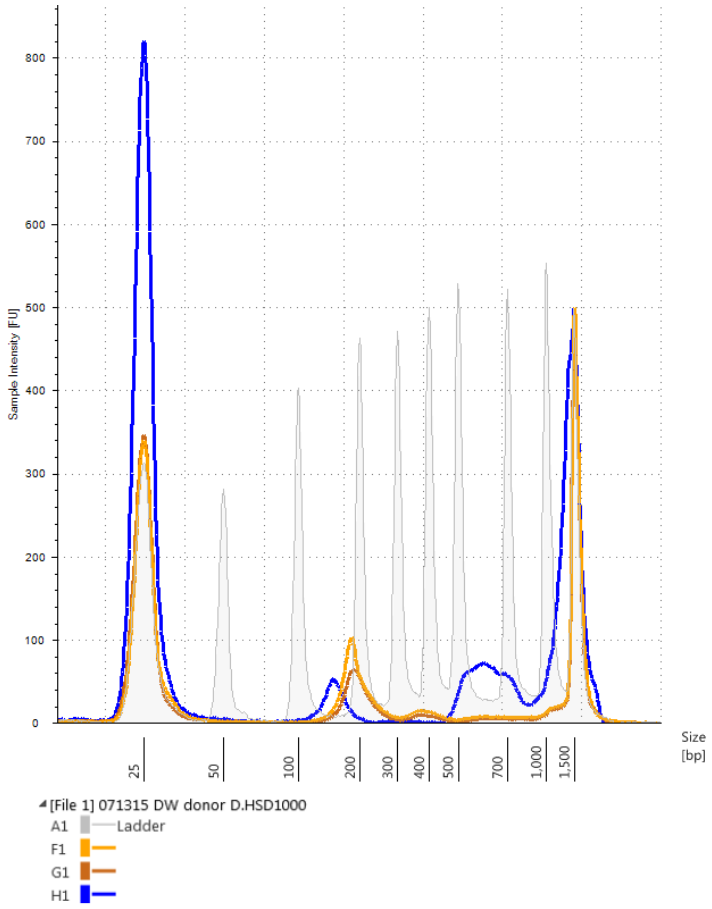
Donor D Run 1

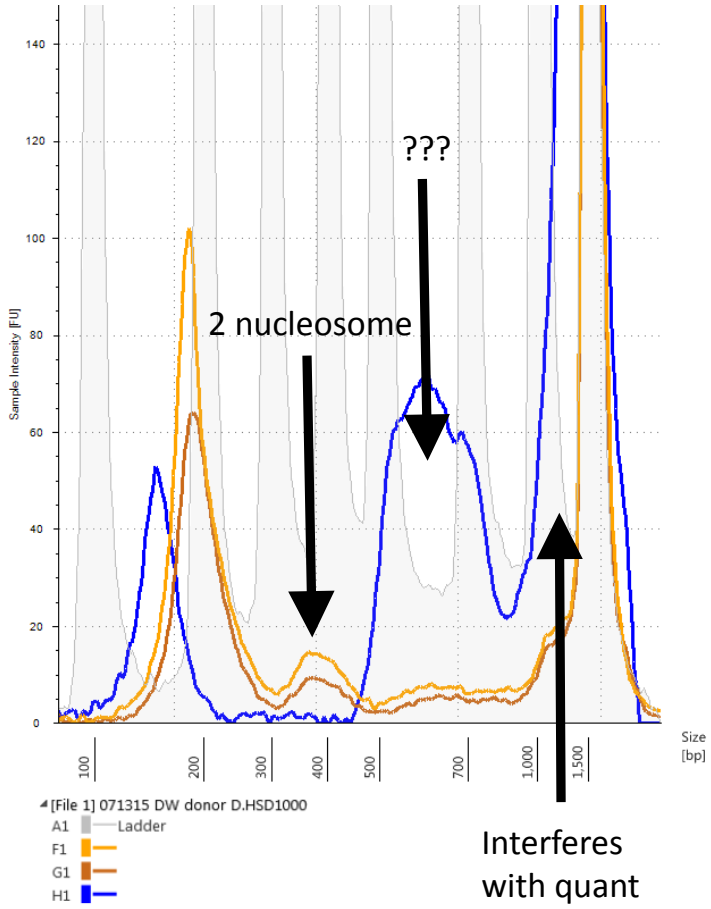


Donor D Run 2



Agilent TapeStation Run





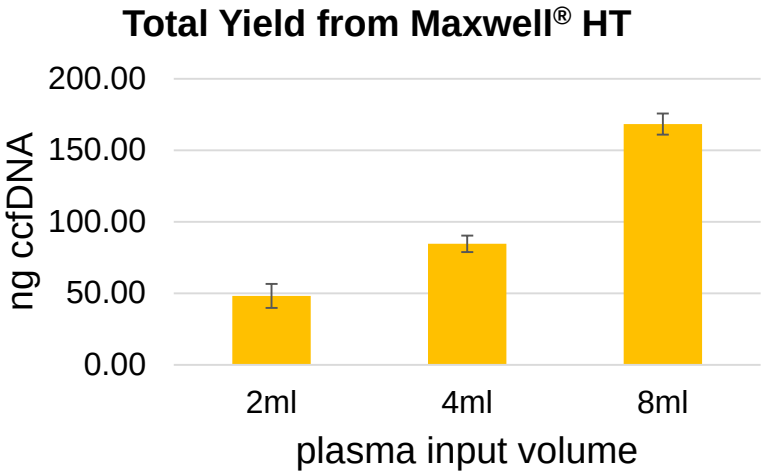
ladder MaxLV MaxHT Qiagen

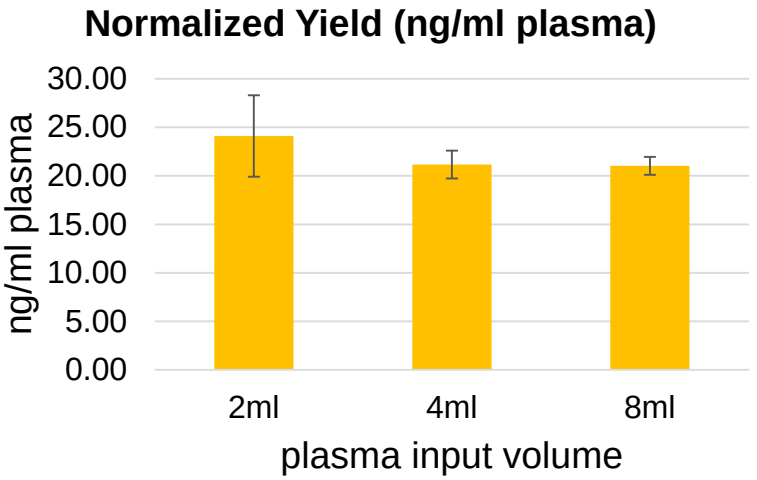
Maxwell® HT System

- Completely automated
- Currently on Hamilton STAR® and Tecan Freedom EVO®
  - Capable of working on any liquid handler
- Can process:
  - 1 to 8mL plasma
  - 1 to 96 samples
- Elute in 50 to 75µL
- Approximate processing times
  One 24-well plate (1 HHS) or
  Four 24-well plates (4 HHS):
  - 2mL 2.5 to 3 hours
  - 4mL 4 to 4.5 hours
  - 8mL 6 to 7 hours

Scalability of Maxwell® HT System

- Increasing amounts of plasma were processed on a Hamilton STAR® using Maxwell® HT chemistry
- 24 samples were processed for each data point
- Samples were eluted in 75µl buffer

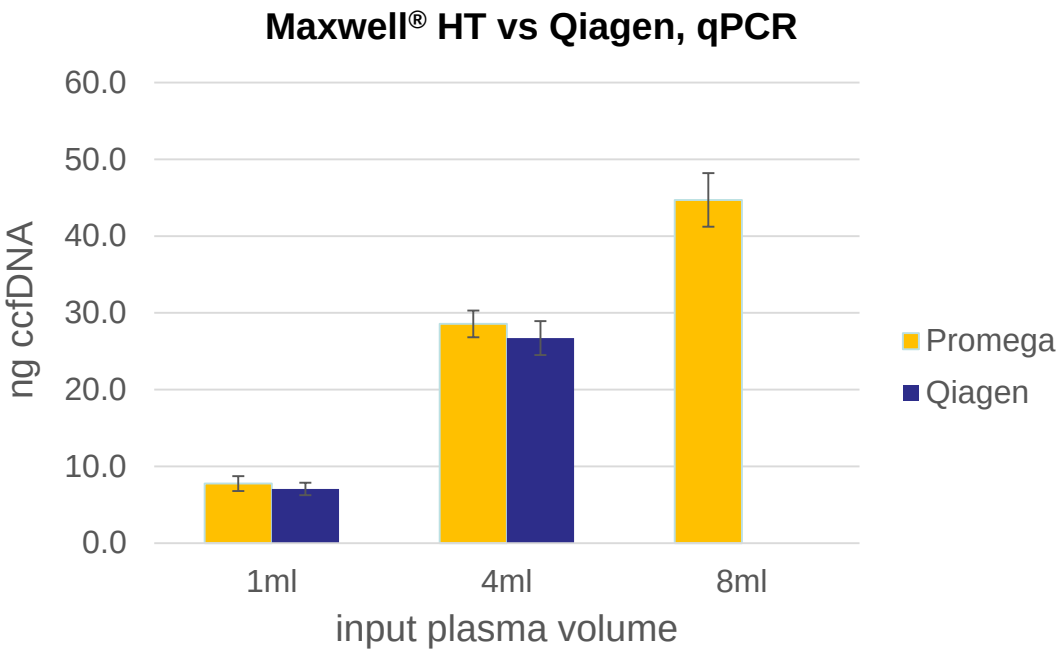




Maxwell® HT vs Qiagen

Plasma from a donor was purified using Maxwell® HT on a Hamilton platform, and Qiagen's manual kit

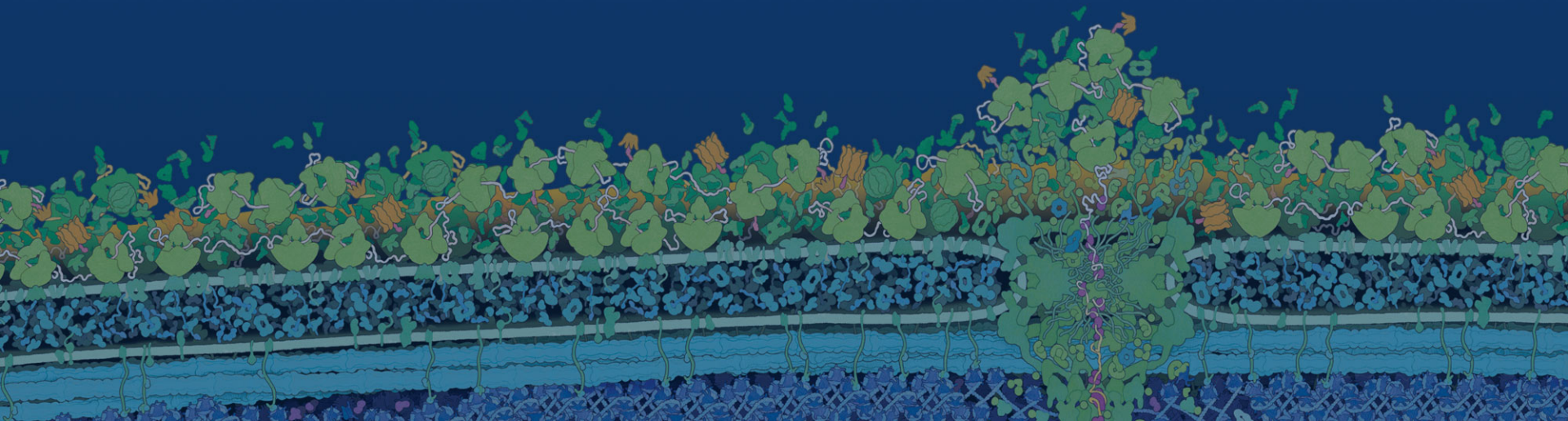
Plasma input volume (n=4 each data point):
Maxwell HT: 1, 4, 8mL
Qiagen: 1, 4mL



- Samples were quantitated using qPCR
- Yields were equivalent at 1 and 4mL
- Qiagen cannot be used to process 8mL

Promega

Quantitation of ccfDNA



Challenges in Quantifying Isolated ccfDNA

- Very low amount means that sensitive detection is a must
- Any genomic DNA carryover is contaminating
- Inhibitor carryover is not seen unless the assay is sensitive to the inhibitor
  - Example 1: Protein contamination
  - Example 2: Alcohol contamination

Methods of Quantifying ccfDNA

- In normal, non-cancerous patients eluate concentrations are typically 0.1-0.5 ng/ul
- UV-VIS determination will not work
- Double stranded DNA specific fluorescent dyes are sensitive enough to quantitate
  - PicoGreen
  - Quantifluor
  - Carrier RNA
- qPCR and ddPCR give results that show amplifiable DNA
- BioAnalyzer or TapeStation results
- Next generation sequencing

Quantitation by Fluorescent Dye

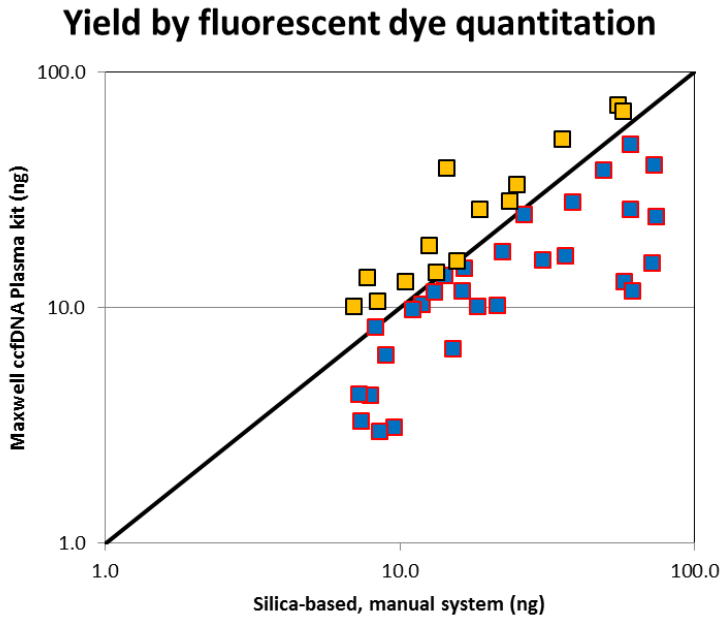
- Sensitive enough for accurate quantitation
  - Accurate to about 0.1ng/ul

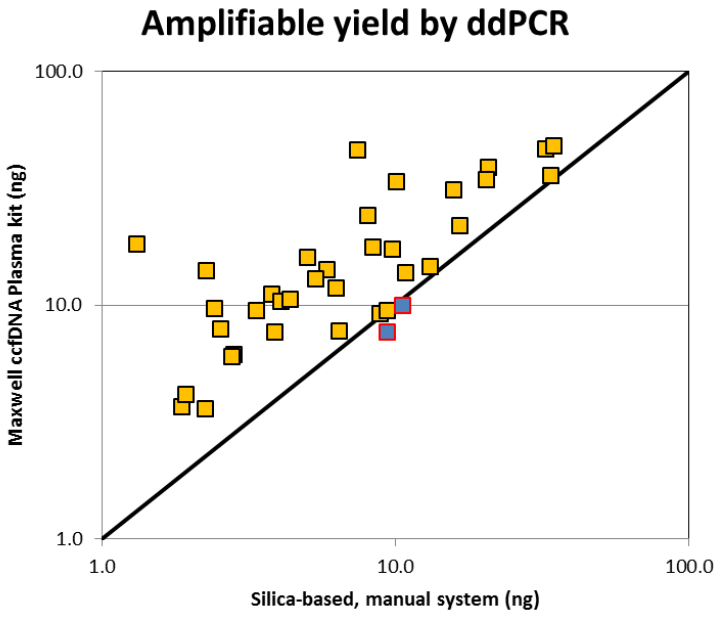
- Some silica systems use a carrier RNA in their preps that interferes with ds-specific dyes
  - Used to improve binding to silica
  - 10ug carrier added; up to 1ug can carry into the elution

- Large amounts of carrier RNA in eluates can inflate the apparent amount of ccfDNA

Fluorescent Detection vs Amplification

- Matched samples run through the Maxwell RSC or silica-based system with carrier RNA

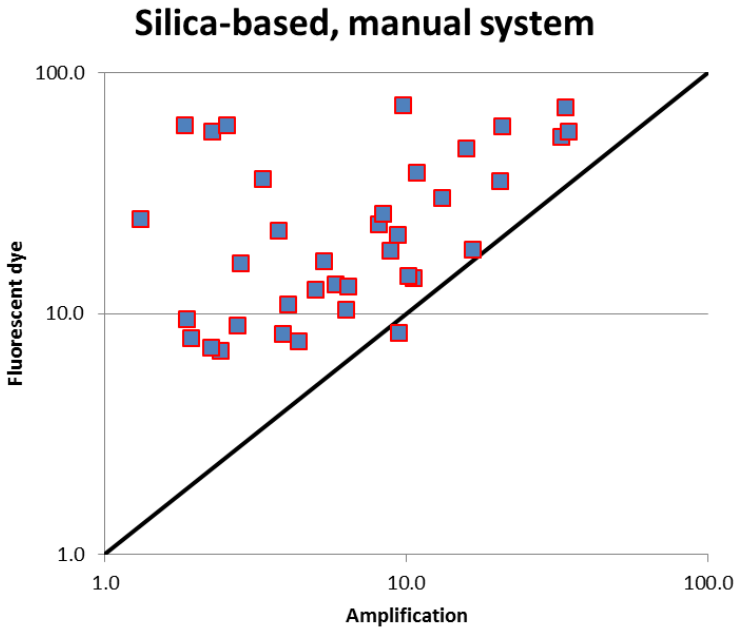


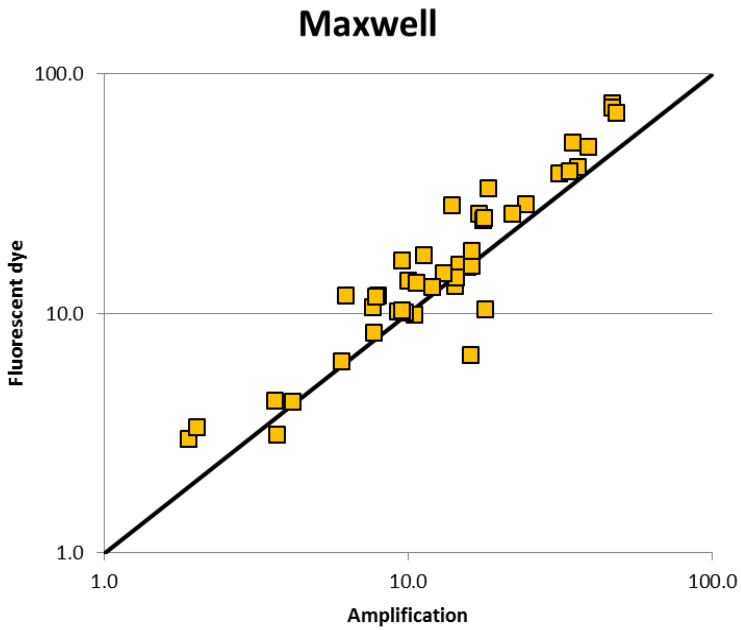


- Silica has more apparent DNA by fluorescent dye
- Maxwell eluates are more amplifiable

Maxwell Eluate Amplification is More Similar to Fluorescent Dye Quantitation than Silica

- Eluate fluorescent dye quantitation compared with digital PCR quantitation





- Silica is heavily skewed towards quant overestimation by dye
- Maxwell eluate quantitation appears more similar between methods

Differential qPCR Quantitation

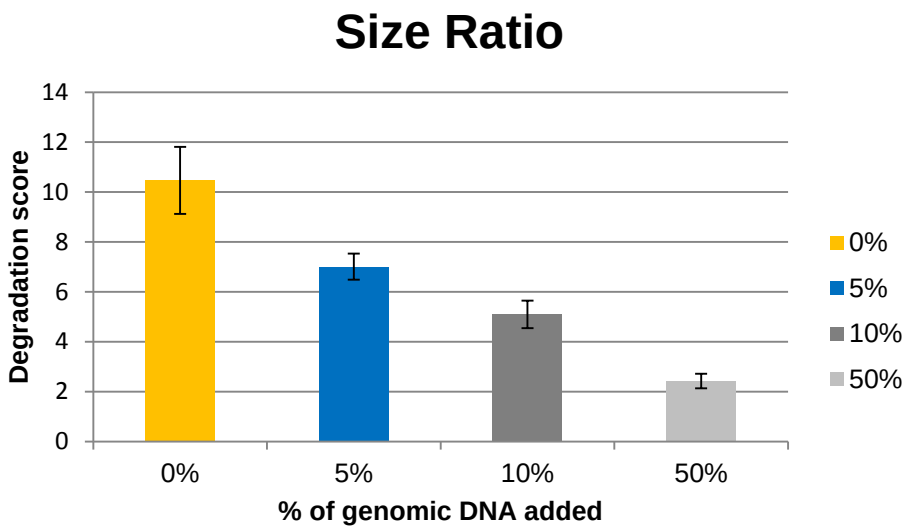
- Provides a way to determine the relative sizes of purified DNA

- Contains 2 amplicons to measure autosomal DNA:
  - Short: 84bp
  - Long: 294bp

- Ratio between the two provides a sizing ratio that measures how fragmented the DNA is

For ccfDNA, This System May Be an Effective QC Prior to Mutation Detection

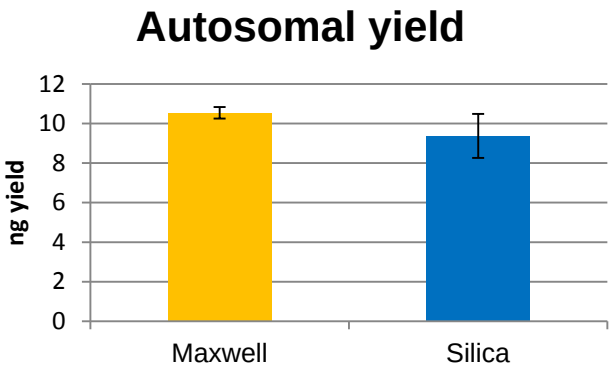
- A representative ccfDNA sample was spiked with gDNA
- Amplification was run to assay for size ratio

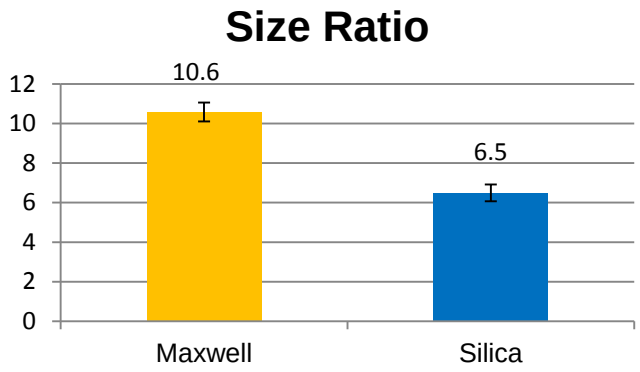


- Increased gDNA contamination results in a lower size ratio
- A low size ratio can indicate purification of larger genomic DNA
- Additional wtDNA from hemolysis of white blood cells can cause problems in mutation detection of ccfDNA

ccfDNA Differential qPCR

Using matched plasma, tested the Maxwell® ccfDNA chemistry vs a manual, competitor system.

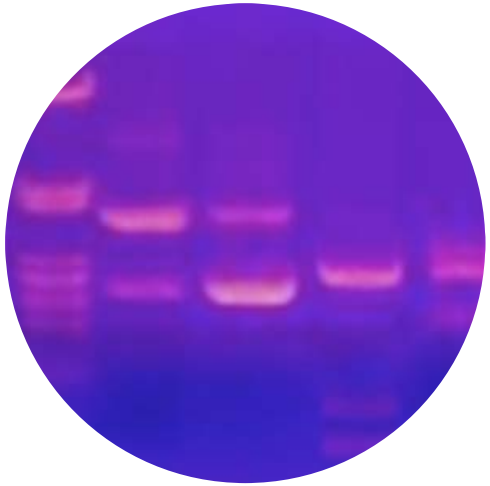
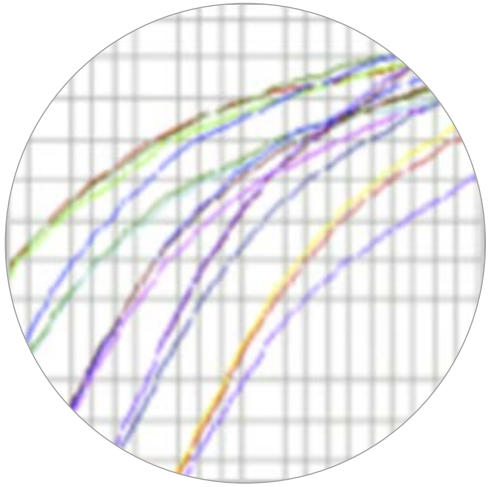
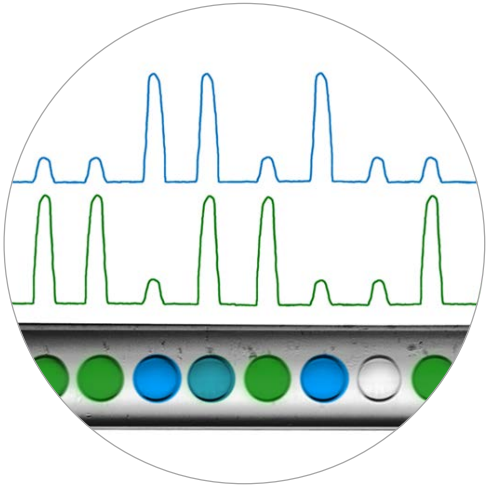




- Starting material was the same
- Yields are equivalent
- Size ratio is lower for silica, suggesting larger fragments of DNA

- ProNex DNA QC Assay

Droplet Digital PCR – The 3rd Generation of PCR

1987	1996	2010
		
PCR Qualitative	**Real-time PCR** Relative Quantification	**Droplet Digital PCR** Absolute Quantification

Bio-Rad Laboratories, Inc.

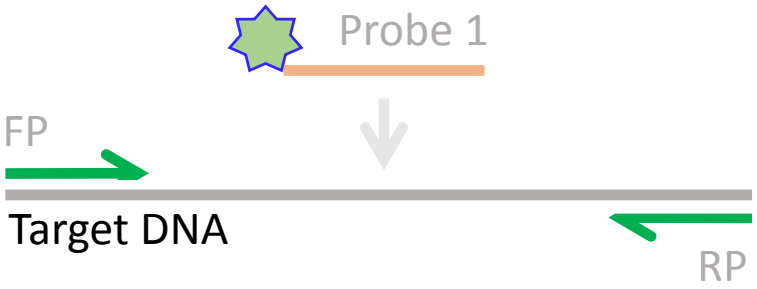
Droplets Enable Thousands of Digital Measurements

One measurement

Nanodroplet PCR reactions are independent, single amplification events

Many thousands of discrete measurements

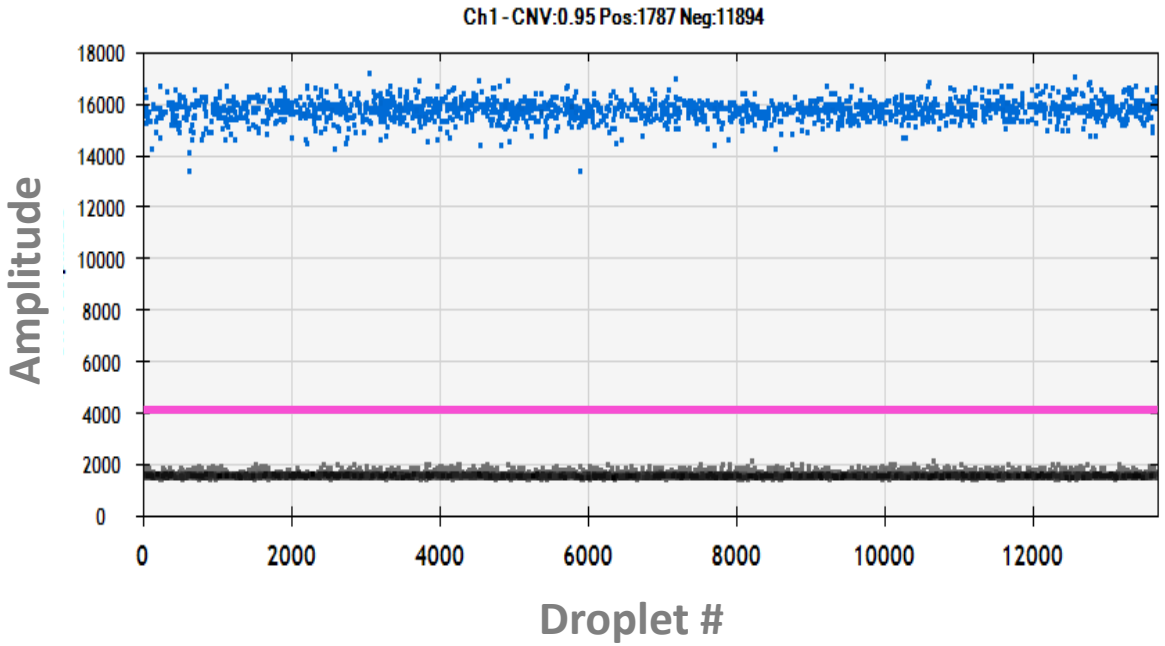
Data Analysis: Singleplex Assay



FAM	-	+

1D Data Analysis: Singleplex Assay

FAM	-	+



Positive Droplets

Threshold

Negative Droplets

Use of Promega ccfDNA and ddPCR

Brain Tumor Pathology
https://doi.org/10.1007/s10014-018-0310-7

ORIGINAL ARTICLE

A novel high-sensitivity assay to detect a small fraction of mutant *IDH1* using droplet digital PCR

Masaki Hirano[1] · Fumiharu Ohka[1] · Sachi Maeda[1] · Lushun Chalise[1] · Akane Yamamichi[1,2,3] · Kosuke Aoki[1] · Akira Kato[1] · Kuniaki Tanahashi[1] · Kazuya Motomura[1] · Yusuke Nishimura[1] · Masahito Hara[1] · Keiko Shinjo[2] · Yutaka Kondo[2] · Toshihiko Wakabayashi[1] · Atsushi Natsume[1]

Received: 4 February 2018 / Accepted: 14 February 2018

nature COMMUNICATIONS

ARTICLE

Received 9 Dec 2015 | Accepted 23 Oct 2016 | Published 8 Dec 2016

DOI: 10.1038/ncomms13665 OPEN

Acquired *RAS* or *EGFR* mutations and duration of response to EGFR blockade in colorectal cancer

Beth O. Van Emburgh[1,2,3,*], Sabrina Arena[1,3,*], Giulia Siravegna[1,3], Luca Lazzari[1,3], Giovanni Crisafulli[1,3], Giorgio Corti[1], Benedetta Mussolin[1], Federica Baldi[1,3], Michela Buscarino[1], Alice Bartolini[1], Emanuele Valtorta[4], Joana Vidal[5,6], Beatriz Bellosillo[6,7], Giovanni Germano[1], Filippo Pietrantonio[8], Agostino Ponzetti[9], Joan Albanell[5,6], Salvatore Siena[4,10], Andrea Sartore-Bianchi[4], Federica Di Nicolantonio[1,3], Clara Montagut[5,6] & Alberto Bardelli[1,3]

www.impactjournals.com/oncotarget/ Oncotarget, 2017, Vol. 8, (No. 49), pp: 86253-86263

Research Paper

Liquid biopsy in colon cancer: comparison of different circulating DNA extraction systems following absolute quantification of *KRAS* mutations using Intplex allele-specific PCR

Vera Kloten[1], Nadine Rüchel[1], Nadina Ortiz Brüchle[1], Janina Gasthaus[1], Nils Freudenmacher[1,2], Florian Steib[1], Jolein Mijnes[1], Julian Eschenbruch[1], Marcel Binnebösel[3], Ruth Knüchel[1] and Edgar Dahl[1,2]

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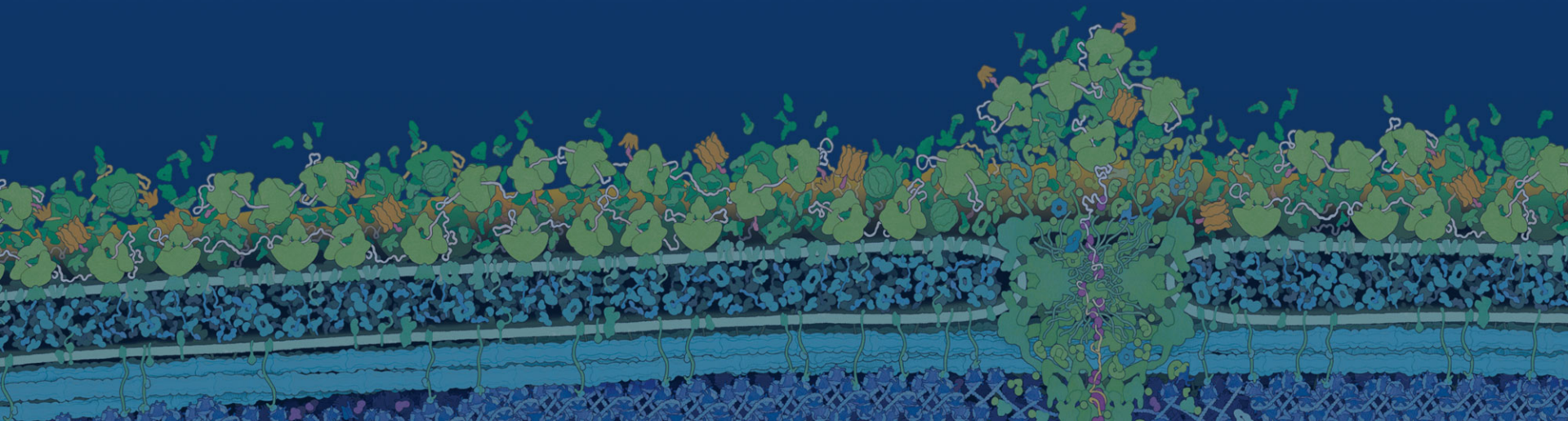
Quantitation Summary

- No UV-VIS
- Fluorescent double stranded DNA specific dyes work well
  - Carrier RNA and genomic DNA can inflate the quantitation
- Best choice is qPCR or ddPCR
- TapeStation results also valid, but are influenced by carrier RNA or gDNA

Comparisons in the Literature



How are evaluations typically done?

- Comparison of multiple kits
- Current lab QC
- Downstream assays used

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A Comparison of Cell-Free DNA Isolation Kits

Isolation and Quantification of Cell-Free DNA in Plasma

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http://dx.doi.org/10.1016/j.jmoldx.2016.09.009

Conclusion of Sorber et al.

*"This study presents two **highly efficient isolation kits**, namely, the QIAamp circulating nucleic acid kit (QIA) and the Maxwell RSC ccfDNA Plasma Kit (RSC), of which the **RSC kit has the advantage of a fully automated, magnetic bead-based protocol** over the labor-intensive QIA kit."*

http://dx.doi.org/10.1016/j.jmoldx.2016.09.009

Original Article

Comparison of methods for circulating cell-free DNA isolation using blood from cancer patients: impact on biomarker testing

Clara Pérez-Barrios[1*], Irene Nieto-Alcolado[2*], María Torrente[3], Carolina Jiménez-Sánchez[2], Virginia Calvo[3], Lourdes Gutierrez-Sanz[3], Magda Palka[3], Encarnación Donoso-Navarro[1], Mariano Provencio[3], Atocha Romero[2,3]

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Contributions: (I) Conception and design: C Pérez-Barrios, V Calvo, L Gutierrez-Sanz, M Palka, M Provencio, A Romero; (II) Administrative support: M Torrente, M Provencio, E Donoso-Navarro; (III) Provision of study materials or patients: E Donoso-Navarro, C Jiménez-Sánchez, V Calvo, L Gutierrez-Sanz, M Palka, M Provencio, A Romero; (IV) Collection and assembly of data: C Pérez-Barrios, I Nieto-Alcolado, C Jiménez-Sánchez, A Romero; (V) Data analysis and interpretation: C Pérez-Barrios, I Nieto-Alcolado, C Jiménez-Sánchez, A Romero; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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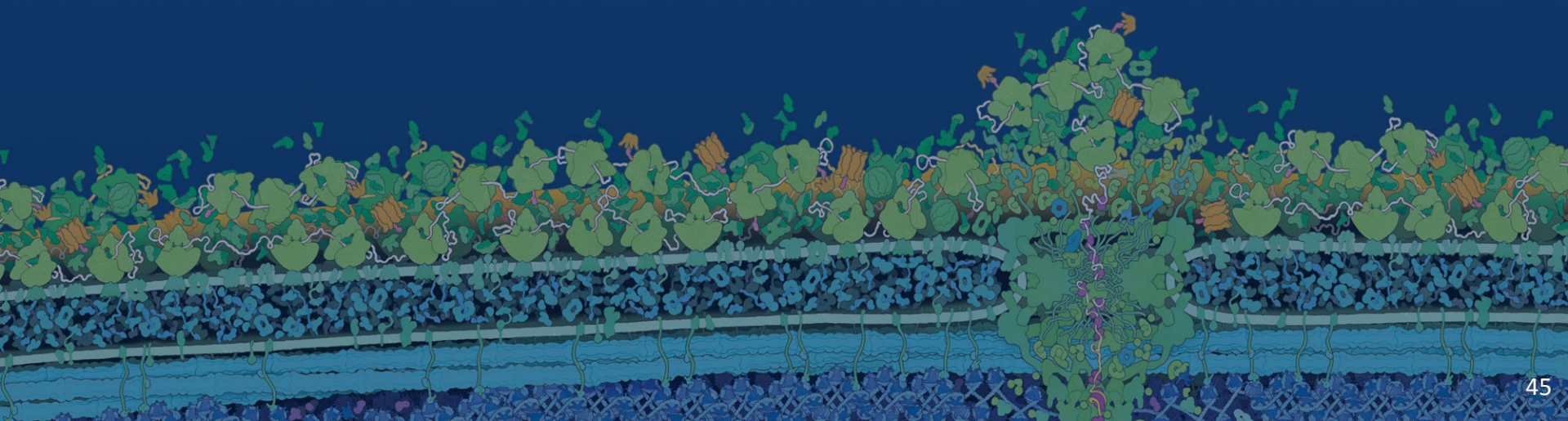
Conclusion of Pérez-Barrios et al.

*"In comparing the QCNA kit and the [**Maxwell® RSC**], we found that the latter was not superior to the former in terms of cfDNA yield, but it **was simpler and more rapid as it is an automated method.**"*

http://dx.doi.org/10.1016/j.jmoldx.2016.09.009

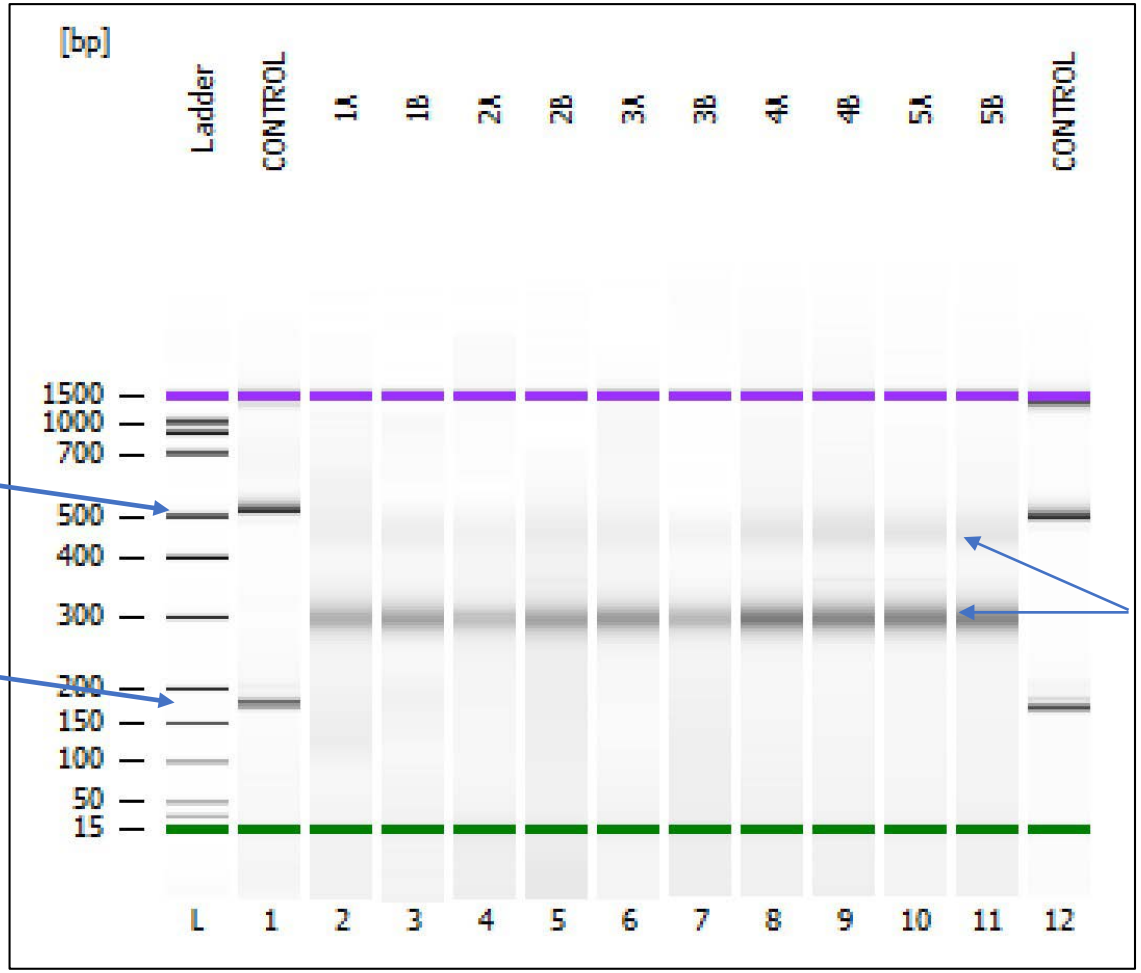
NGS and ProNex® Size-Selective Purification System



Illumina TruSeq DNA Nano LT Kit Steps

1. **End repair and size selection:**
   - Step 1 - subtraction using ProNex® chemistry at 1.1vol:1vol(110ul:100ul)
   - Step 2 - recovery using add'l 190ul ProNex® chemistry, eluting in 17.5ul
2. **Post A-tailing and adapter ligation:**
   - Step 1 - cleanup using ProNex® chemistry at 1.6 vol:1vol (68ul:42.5ul)
   - Step 2 - elute in 50ul
   - Step 3 - repeat cleanup using ProNex® chemistry at 1.6 vol:1vol(80ul:50ul)
   - Step 4 - elute in 25ul
3. **8-cycle PCR enrichment:**
   - Step 1 - repeat cleanup using ProNex® chemistry at 1.6 vol:1vol(80ul:50ul)
   - Step 2 - Elute in 30ul

Bioanalyzer analysis of CCF templates



500bp control

172bp control

170bp and 340bp CCF templates with adapters

Recap

- ccfDNA is a dilute, highly fragmented and transient component of plasma

- We developed a completely automated method on the Maxwell® RSC (and larger automation platforms) for rapid purification of high-quality ccfDNA

- Amplification-based methods are best for quantitation

Promega

Coming soon…

ccfDNA Webinar Series: The Basics and Beyond

Part 3

ccfDNA In the Lab: Optimizing Purification for Sequencing